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Abstract

Influenza A virus mRNAs are transcribed by the viral RNA-dependent RNA polymerase in the cell nucleus before being exported to the cytoplasm for translation. Segment 7 produces two major transcripts: an unspliced mRNA that encodes the M1 matrix protein and a spliced transcript that encodes the M2 ion channel. Export of both mRNAs is dependent on the cellular NXF1/TAP pathway but it is unclear how they are recruited to the export machinery or how the intron-containing but unspliced M1 mRNA bypasses the normal quality control checkpoints. Using fluorescent *in situ* hybridization to monitor segment 7 mRNA localisation, we found that cytoplasmic accumulation of unspliced M1 mRNA was inefficient in the absence of NS1, both in the context of segment 7 RNPs reconstituted by plasmid transfection and in mutant virus-infected cells. This effect was independent of any major effect on steady-state levels of segment 7 mRNA or splicing, but corresponded to a ~ 5-fold reduction in the accumulation of M1. A similar defect in intronless HA mRNA nuclear export was seen with an NS1 mutant virus. Efficient export of M1 mRNA required both an intact NS1 RNA-binding domain and effector domain. Furthermore, while wildtype NS1 interacted with cellular NXF1 and also increased the interaction of segment 7 mRNA with NXF1, mutant NS1 polypeptides unable to promote mRNA export did neither. Thus we propose that NS1 facilitates late viral gene expression by acting as an adaptor between viral mRNAs and the cellular nuclear export machinery to promote their nuclear export.

45 **Importance**

46 Influenza A virus is a major pathogen of a wide variety of mammalian and avian species that
47 threatens public health and food security. A fuller understanding of the virus life cycle is
48 important to aid control strategies. The virus has a small genome that encodes for relatively few
49 proteins that are often multifunctional. Here, we characterise a new function for the NS1 protein,
50 showing that as well as previously identified roles in antagonising the innate immune defenses of
51 the cell and directly upregulating translation of viral mRNAs, it also promotes the nuclear export
52 of the viral late gene mRNAs by acting as an adaptor between the viral mRNAs and the cellular
53 mRNA nuclear export machinery.

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Introduction

Influenza A virus (IAV) has a genome constituted of eight single-stranded, negative-sense RNA molecules, each separately encapsidated into ribonucleoprotein (vRNP) particles with one copy of the viral PB1:PB2:PA heterotrimeric RNA polymerase and multiple copies of a nucleoprotein (NP) (1). These RNPs are the templates for transcription and replication of the genome (the latter by a cRNA replicative intermediate), which occurs in the host cell nucleus. Use of this cellular compartment provides access to the mRNA splicing machinery and potentially avoids cytoplasmic viral RNA sensors such as RIG-I, but introduces the need for nuclear export of both replicated genomic vRNA (as vRNPs) and viral messenger RNA. Nuclear export of IAV vRNPs is well characterised; the CRM1 cellular pathway is accessed by the participation of two further viral proteins: M1 and NS2/NEP, with NS2 acting as an adaptor protein betweeen vRNP-bound M1 and CRM1 itself (2, 3). The mechanism underlying IAV mRNA export however remains less well understood, further complicated by the fact that there are different types of transcripts to consider: intronless (from segments 1-6), intron-containing but unspliced (segments 7 and 8, mRNAs encoding M1 and NS1 respectively) and their fully spliced counterparts (for segment 7, either mRNA 2 encoding M2 or a so far hypothetical 9mer peptide encoded by mRNA3 and NS2 from spliced segment 8 mRNA [(4)]). These mRNAs can additionally be divided into early (segments 1-3, 5 and unspliced 8) and late (segments 4, 6, unspliced 7 and the two spliced transcripts) classes, according to the expression kinetics of the proteins they encode (4, 5), raising the possibility that different strategies might be used for specific mRNAs. All mRNA transcripts nevertheless start with a host-derived $^{7m}GpppG_m$ cap structure derived from cellular pre-mRNAs by the process of "cap-snatching" (1) which provides structural identity between the 5'-ends of viral and cellular mRNAs.

79 Several of the viral late gene mRNAs have been shown to be retained in the nucleus in the
80 presence of inhibitors of RNA polymerase (Pol) II (6-8), suggesting that they normally use a
81 cellular pathway for nuclear export. Subsequent work has identified this as the cellular NXF1
82 (TAP)-dependent pathway, the route by which the majority of cellular mRNAs reach the
83 cytoplasm (9, 10). RNAi silencing of NXF1 in human embryonic kidney 293 cells inhibited viral
84 gene expression and overall replication (11) and was subsequently shown to reduce nuclear
85 export of all influenza mRNAs tested, albeit with a gradient of sensitivity in which the M1
86 mRNA was the most sensitive and NP mRNA the least (12, 13). Furthermore, IAV mRNAs from
87 segments 2, 4, 5, 6 and 7 have been shown to co-precipitate with the cellular nuclear cap-binding
88 protein and/or NXF1 (13-15), further confirming use of the cellular mRNA export pathway.

89 Cellular mRNAs are recruited to the NXF1 pathway co-transcriptionally, by the
90 deposition of various cellular factors (the transcription-export or TREX complex) onto the
91 nascent mRNA after addition of the 5'-cap structure as well as during the removal of introns (16-
92 19) . The Aly component of TREX then 'hands over' to NXF1 and its co-factor p15, which then
93 interact with the nuclear pore to direct export of the transcript (20). However, since all influenza
94 mRNAs are synthesized by the viral polymerase rather than by RNA Pol II, and most either do
95 not contain introns or are exported with unprocessed introns, it is not clear how they are recruited
96 to the NXF1 pathway. Herpesviruses have solved the problem of exporting intronless viral
97 mRNAs by providing a viral adaptor protein to interact with Aly and thus recruit the TREX
98 complex components needed for export (21). The IAV NS1 protein has been hypothesised to
99 perform an analogous function, based on its known interactions with various cellular mRNA
100 processing and export factors as well as with viral mRNAs (22). Consistent with this, a recent
101 study defined a role for NS1 in recruiting segment 7 mRNA to nuclear speckles for splicing, but

also for subsequent nuclear export (23). To further define the export pathways used by IAV mRNAs, we examined the minimum requirements for nuclear export of viral mRNAs transcribed by reconstituted RNPs, focusing on segment 7 mRNAs. We found that they are largely retained in the nucleus in the absence of the viral NS1 protein and that while wild type (WT) NS1 bound both viral mRNA and NXF1 and promoted the interaction of segment 7 mRNA with NXF1, mutant NS1 proteins that had lost these activities failed to support efficient export of viral mRNA. Furthermore, HA mRNA was inefficiently exported in the absence of a fully functional NS1 protein. Thus we conclude that the NS1 protein acts as an adapter molecule to direct viral late gene mRNAs to the cellular nuclear export machinery.

Results

Synthetic viral late gene mRNAs are inefficiently exported in the absence of NS1

Influenza A virus transcription occurs in the nucleus of infected cells which necessitates that viral mRNAs be exported to the cytoplasm. This is achieved at least in part by use of the main cellular mRNA NXF1/TAP-dependent export pathway (8, 11-13, 15, 23). However, transcripts from individual segments show differential requirements for the cellular export machinery (8, 12, 13) and it is not clear how the viral mRNAs are fed into the cellular export pathway. To pursue the hypothesis that a viral polypeptide acts as an adapter between the viral transcription machinery and the cellular export pathway, we compared the localization of individual viral mRNAs in the context of virus infection and in the context of an RNP reconstitution system, reasoning that the latter approach might reveal a role for a viral component other than the minimal requirements of the three polymerase subunits (3P) and NP

needed for transcription in a 'minireplicon' assay. We focused on segment 7 mRNAs because prior work had indicated a strong dependency on the cellular NXF1 pathway for their nuclear export (8, 12, 15).

When segment 7 mRNA localization was observed by fluorescence *in situ* hybridization (FISH) of virus-infected 293T cells at 6 h p.i. (using a probe complementary to both unspliced M1 and spliced M2 mRNAs), the majority of the transcripts were cytoplasmic (Fig 1A), as expected (8, 12, 23). Time course experiments showed substantial cytoplasmic accumulation of segment 7 mRNA from as early as 4.5h p.i. (data not shown). However, when cells were transfected with 3P and NP expression plasmids and a plasmid encoding segment 7 vRNA under an RNA Polymerase I promoter (Pol I) to reconstitute segment 7 RNPs, the transcripts showed marked (although not total) nuclear retention at 24h post transfection (Fig 1B). The negative controls for both infection (mock infected cells) and transfection (lacking the PB2 subunit of the polymerase) gave no significant signal, showing the specificity of the probe used. Thus segment 7 mRNAs were not exported efficiently in the RNP reconstitution system, suggesting the normal involvement of a viral factor coming from a gene not included in the minimal set needed to recreate an RNP.

Next, the transfected minimal segment 7 transcriptional unit was supplemented with additional Pol I plasmids that expressed each of the 'missing' vRNAs (segments 4, 6 and 8) and segment 7 mRNA localization observed as before by FISH. Again, positive sense transcripts from reconstituted segment 7 RNPs alone were largely nuclear (Fig 2A). The addition of either segment 4 or segment 6 (and thus the expected expression of HA or NA respectively) did not alter segment 7 mRNA localization. Addition of segment 8 did substantially alter the staining pattern however, with many more cells showing markedly greater amounts of cytoplasmic

148 staining. When replicate experiments were scored for the number of cells showing
149 predominantly nuclear, predominantly cytoplasmic or a mixed pattern of segment 7 mRNA
150 localization, the addition of segment 8 but not segment 4 caused a clear shift towards
151 cytoplasmic localisation (Fig 2B), indicating that a segment 8 gene product promotes segment 7
152 mRNA export.

153 Segment 8 of PR8 encodes two identified proteins: NS1, produced from the unspliced
154 mRNA transcript and NS2/NEP, from a spliced mRNA (24, 25). To distinguish between the
155 effects of NS1 and NS2, plasmids expressing either influenza A NS1 or influenza A NS2
156 proteins were transfected together with segment 7 and 3PNP. As a further control, we also tested
157 NS1 from influenza B virus (NS1B). In addition, because segment 7 produces spliced and
158 unspliced mRNAs (26, 27), the cells were hybridized with an intron-specific probe specific for
159 M1 mRNA as well as with the pan-segment 7 probe. Both probes indicated a predominantly
160 nuclear localization for all detectable positive-sense segment 7 RNA species when segment 7
161 RNPs were reconstituted alone (Fig 2C). NS2 protein alone was not capable of rescuing segment
162 7 mRNA export, as the mRNA was visibly still retained in the nuclei of most cells (Figs 2B, C).
163 Similarly, the addition of NS1B did not result in segment 7 mRNA export. In contrast, addition
164 of NS1 from IAV had a clear effect on mRNA localization, as the majority of cells now
165 displayed mostly cytoplasmic fluorescence with both segment 7 probes.

166 Protein expression of the segment 7 and 8 gene products was analyzed by western
167 blotting, confirming the expression of NS1, NS2 and NS1B in the expected samples (Fig 2D,
168 lanes 5-8; note the presence of V5 epitope tags on NS1 and NS1B proteins). NP levels were
169 comparable between samples, suggesting similar transfection levels, while examination of
170 tubulin levels confirmed equal gel loading. M1 and M2 proteins were not detected in the

171 negative control where segment 7 transcription was blocked by the omission of PB2 (lane 1).
172 Otherwise M1 and M2 proteins (the latter running as a doublet, possibly because of post-
173 translational modification [(28)]) were detected in all conditions where a segment 7 RNP was
174 reconstituted (lanes 2-8). Notably however, the expression levels for M1 and M2 were higher
175 when either segment 8 or NS1 were added (lanes 5 and 6). This increase in M1 and M2
176 accumulation in the presence of NS1 is consistent with the more efficient release of segment 7
177 mRNA to the cytoplasm seen by FISH, as well as with the ability of NS1 to increase translation
178 of viral mRNAs.

179 In some (but not all) studies, NS1 expression has been found to affect the extent of
180 segment 7 splicing (23, 29-31). It was therefore conceivable that, if the M1 and M2 mRNAs
181 have intrinsic differences in transport efficiency, NS1 could indirectly promote segment 7
182 mRNA export by changing the balance between spliced and unspliced products. To test this
183 hypothesis, segment 7 mRNA splicing was analysed using radiolabelled primer reverse
184 transcription. In our A/PR/8/34 (PR8)-based system, M1 and M2 mRNAs accumulated to
185 approximately equal amounts in the absence of NS1, while mRNA3 formed a minority species
186 (Fig 3A). The levels of genomic vRNA were also reasonably consistent. However, expression of
187 NS1 had variable and generally modest effects on the overall levels of segment 7 splicing, with
188 on average mRNA2 remaining slightly more abundant than mRNA1 (Fig 3B). The most
189 consistent effect, of a slight supression of splicing was in fact seen with NS2, which did not
190 promote cytoplasmic accumulation of the transcripts. In all cases, NS1 (or NS2) expression
191 changed the relative abundance of the individual segment 7 mRNA species by less than 2-fold.
192 Overall therefore, this indicated that the PR8 NS1 protein promotes efficient nuclear export of
193 segment 7 mRNAs without major effects on their differential splicing.

194 NS1 protein has two functional domains, an effector domain and an RNA-binding
195 domain, each associated with several functions (32). To assess whether the ability of NS1 to
196 promote segment 7 mRNA export was intrinsic to one of the domains, a set of NS1 mutants were
197 generated fused with a GFP tag. As before, each of the NS1-GFP mutants were added separately
198 to the segment 7 RNP reconstitution assay and segment 7 mRNA localization analyzed by FISH
199 24 h later. As expected, segment 7 mRNA could not be detected in the 2PNP negative control
200 and when transcribed in the absence of any additional non-RNP influenza A proteins, it was
201 found in the nucleus, as well as when made in the presence of NS2-GFP (Fig 4A; quantification
202 in Fig 4B). In contrast, when NS1-GFP was also transfected, the mRNA was efficiently exported
203 to the cytoplasm, indicating that the addition of a GFP-tag did not block the export-promoting
204 activity of NS1. An NS1-GFP mutant in which Cleavage/polyadenylation specificity factor 30
205 (CPSF)-inhibitory activity had been restored by appropriate mutation of effector domain residues
206 S103 and I106 ((S+I)-GFP; (33, 34)) also promoted the efficient export of segment 7 mRNA.
207 However, when a mutant NS1 consisting of only the RNA-binding domain (N81-GFP; where a
208 stop codon was inserted at codon 82) was co-transfected, the mRNA was found largely retained
209 in the nucleus. Similarly, an RNA-binding domain mutant (R+K)-GFP with charge to alanine
210 mutations in residues R38 and K41 was not capable of releasing segment 7 mRNA from the
211 nucleus of the majority of transfected cells. Thus both functional domains of NS1 protein are
212 required to promote export of segment 7 mRNA. Expression of each GFP construct was
213 confirmed by confocal microscopy (Fig 4A) as well as by western blot analyses (Fig 4C), with
214 the latter approach also showing increased expression of M1 and M2 polypeptides in the
215 presence of the fully export-competent NS1 polypeptides and an intermediate phenotype from
216 the N81 or R+K mutants.

NS1 is required for efficient M1 mRNA export in infected cells

To test whether NS1 was required for efficient segment 7 mRNA export in the context of a viral infection, NS1 mutant viruses were generated by reverse genetics, with none of the mutations affecting the NS2 gene. Further single residue RNA-binding domain mutants (NS1-R38A and NS1-K41A) were generated in addition to the double mutant NS1-(R+K). Cells were infected with WT PR8 or the NS1 mutant viruses and segment 7 mRNA localization was observed by FISH at 6 h.p.i.. Segment 7 mRNA was not detected in mock-infected cells and was found in the cytoplasm in WT-infected cells as observed previously (Figure 5A). A similar outcome was obtained with the CPSF-binding site mutant virus NS1-(S+I). However, in agreement with the findings from RNP reconstitution assays, neither the R+K RNA binding domain mutant nor the N81 effector domain mutant supported normal segment 7 mRNA export. When replicate experiments were imaged and scored for the proportion of cells showing nuclear retention of segment 7 mRNA, around 70-80% of cells infected with the R+K or N81 mutants showed this pattern, in contrast to the overwhelmingly cytoplasmic phenotype of WT or S+I mutant infected cells (Fig 5B). Analysis of single residue RNA-binding mutants showed an intermediate effect, with some cells supporting apparently normal mRNA export, others showing nuclear retention (Figs 5A, B). Western blot analysis of infected cell lysates confirmed expression of all NS1 polypeptides with the exception of the truncated protein produced by the NS1-N81 mutant, which could not be detected by the effector domain-specific antiserum used here (Fig 5C and data not shown; the trace amount of apparently full length NS1 detectable in lane 3 likely reflects low levels of reversion in the virus stock). NP and NS2 expression levels were consistent between all the viruses, but the accumulation of segment 7-derived polypeptides

showed a clear correlation with the localisation of the mRNA, with efficient nuclear export leading to higher levels of M1 and M2 synthesis (Fig 5C). Quantification of M1 and M2 accumulation from replicate experiments showed that poor nuclear export of segment 7 mRNA lead to around a 3-5 fold reduction in the quantity of M1 relative to NP and 2-3 fold reductions in the amount of M2 (Fig 5D). Thus in infection as well as in transfection, a functional NS1 protein is required to promote efficient nuclear export and expression of segment 7 mRNA.

As a further test of the hypothesis that NS1 might promote export of segment 7 mRNA through its effects on the cellular splicing machinery, we asked whether a drug that inhibits splicing affected mRNA export in the presence or absence of a functional NS1 protein. The SF3b inhibitor, spliceostatin A has been shown to potently inhibit pre-mRNA splicing and consequently suggested to allow the passage of intron-containing transcripts to the cytoplasm (35). Cells were infected with either WT or NS1-N81 viruses or mock infected and duplicate samples treated with spliceostatin A. At 6 h.p.i., segment 7 mRNA cellular localization was determined by FISH. As before, the mRNA was cytoplasmic in WT infection and largely retained in the nucleus after infection by the N81 virus, but neither outcome was changed by the addition of the drug (Fig 6A). Analysis of viral protein expression by western blot confirmed that M1 and NS1 (produced from the unspliced transcripts of segment 7 and 8, respectively) were expressed with or without drug but that spliced products M2 and NS2 were only detected in non drug-treated infected cells (Figure 6B, compare lanes 2 and 3 with 5 and 6). Analysis of segment 7 mRNA accumulation by primer extension further confirmed that the drug blocked production of spliced mRNA2 (Fig 6C). Thus the mRNA export function of NS1 is independent of mRNA splicing, either as a positive or negative factor.

NS1 interacts with NXF1 and viral mRNAs to promote their export.

We next tested the hypothesis that NS1 protein acted as an adaptor protein to deliver the viral mRNA to the cellular mRNA export machinery. This hypothesis is consistent with the dependence of segment 7 transcripts on the cellular NXF1 pathway for export (12) as well as with interactions between influenza A mRNAs and both NS1 and NXF1 and between NS1 and NXF1 themselves (13, 15, 36, 37). First, we examined whether mutant NS1 proteins that failed to promote segment 7 mRNA export bound NXF1. 293T cells were transfected with either GFP or GFP-NXF1 and 48 h later, mock infected or infected with WT or the various NS1 mutant viruses. At 6 h.p.i cells were collected, lysed and the supernatants subjected to GFP-trap pull downs. Western blot analyses of total and bound fractions showed that GFP-NXF1 and GFP were expressed as expected and bound well to the affinity matrix (Fig 7). NS1 was present in all infected samples, including the truncated N81 protein although detection of this last polypeptide required use of an RNA-binding domain-specific antiserum and was inefficient (lanes 1,2 and 4-8). Consistent with previous reports (13, 36), WT NS1 co-precipitated with NXF1, as did the NS1 S+I mutant (lanes 10 and 16). However, none of the NS1 RNA-binding domain mutants bound to detectable levels (lanes 13-15). Similarly, the effector domain deleted N81 protein was not apparent in the bound fraction (lane 12), suggesting that (within the limits of detection of the antibody) the truncated protein did not bind NXF1. Thus there was a good correlation between the ability of NS1 to promote segment 7 mRNA export and its ability to bind NXF1.

Previous work has shown that NS1 binds segment 7 mRNA *in vitro* and co-precipitates it from infected cells (15, 37, 38). We therefore tested whether the strength of this interaction correlated with the ability of NS1 mutants to promote efficient export of M1 mRNA. First, we used the RNP reconstitution assay to recreate segment 7 RNPs with or without the addition of

286 WT or mutant GFP-NS1 polypeptides, before fractionating cell lysates over GFP-trap beads and
287 analysing the amounts of bound segment 7 mRNA. Examination of aliquots of total cell lysate
288 showed the expected presence of M1 and M2 mRNA species in all samples transfected with all
289 four RNP polypeptides but not in 2P control samples (Fig 8, lanes 1-10). No detectable mRNA
290 co-precipitated with GFP, but easily detectable amounts of both mRNA1 and mRNA2 bound to
291 duplicate samples co-transfected with WT GFP-NS1 or S+I GFP-NS1 (lanes 14, 15 and 20).
292 However, the export-incompetent N81, R38, K41 and R+K mutants all failed to detectably bind
293 M1 mRNA and/or bound much reduced amounts of M2 mRNA (lanes 16-19).

294 NXF1 has also been shown to bind M1 mRNA in WT virus infected cells (15). We
295 therefore used the RNP reconstitution system to ask if (as predicted by the adaptor hypothesis)
296 NS1 facilitated this interaction. 293T cells were transfected with the plasmids needed to recreate
297 segment 7 RNPs (or with a 2P negative control) along with GFP-NXF1 and additionally, with or
298 without NS1. 48 h later, cells were lysed and segment 7 mRNA accumulation examined by
299 primer extension before or after GFP-trap affinity purification. Abundant quantities of M1
300 mRNA and lesser amounts of spliced mRNA2 were present in the total cell lysates from the
301 3PNP but not the 2P control samples, while examination of cellular 5S rRNA confirmed the
302 extraction of equal cell numbers (Fig 9A, lanes 1-3). No detectable M2 mRNA (or 5S rRNA) and
303 only trace quantities of M1 mRNA co-purified with GFP-NXF1 in the absence of NS1 (lane 5).
304 However, both viral mRNAs were readily detectable in samples containing GFP-NXF1 and NS1
305 (lane 6). Thus NS1 promotes the stable interaction of NXF1 and segment7 mRNA.

306 Next, we correlated the ability of mutant NS1 proteins to promote NXF1-segment 7
307 mRNA interactions with their mRNA export activity. 293T cells were transfected with either
308 GFP or GFP-NXF1 and 48h later, infected with the panel of WT and NS1 mutant viruses. At 6 h

p.i., total RNA was extracted before or after the lysates had been subjected to GFP-trap pull downs and primer extension reactions carried out to assay segment 7 mRNAs and vRNA, as well as 5S rRNA as a loading control. Viral RNAs were detected in the total fraction of every infected sample (Fig 9B, lanes 1-7) but only 5S rRNA was detected in mock infected cells (Figure 7A, lane 8). Analysis of the bound fractions from WT virus-infected cells showed that both segment 7 mRNA species co-precipitated with GFP-NXF1 (lane 10), agreeing with a previous study (15). This interaction was specific since genomic vRNA did not precipitate with GFP-NXF1 (lane 10) while none of the viral RNA species bound to GFP only (lane 9). A similar outcome was obtained with the export-competent NS1 (S+I) mutant virus (lane 15). However, only trace quantities of M1 or M2 mRNAs bound NXF1 in cells infected with the N81 or R+K NS1 mutant viruses (lanes 11 and 14), while reduced amounts co-precipitated from cells infected with the single RNA-binding domain R38 and K41 mutants (lanes 12 and 13). Thus the ability of NS1 polypeptides to direct efficient export of segment 7 mRNA showed a strong correlation with their ability to promote the interaction of the transcripts with NXF1, consistent with the adaptor protein hypothesis.

Finally, we asked if NS1 plays a similar role in promoting the nuclear export of other viral mRNAs. Prior studies indicate that nuclear export and/or expression of the other late gene mRNAs for HA and NA show similar sensitivities to the M gene mRNAs to treatment with inhibitors of RNA Pol II, whereas the early class RNP genes do not (7, 8, 12, 15, 39, 40). We therefore compared the intracellular localisation of the PB1 polymerase gene (segment 2), NP (segment 5) and HA (segment 4) mRNA in cells infected with WT or the NS1 N81 mutant virus by FISH. Positive sense segment 5 RNA was almost exclusively detected in the cytoplasm of cells infected with either virus (Fig 10), indicating that unlike segment 7 mRNAs, NP mRNA

nuclear export does not require an intact NS1 polypeptide. Segment 2-specific signal was most prominent in the nuclei of infected cells, perhaps reflecting proportionally greater detection of cRNA from a segment where the two classes of positive sense RNA are present in similar amounts (41, 42). However, the levels of cytoplasmic staining seen in the WT infection were not noticeably diminished in cells infected with the NS1-N81 virus, suggesting that segment 2 mRNA export is also NS1-independent. In contrast, HA mRNA localisation altered from almost totally cytoplasmic in WT infected cells to marked nuclear retention in NS1-N81-infected cells. Thus NS1 has a role in promoting the efficient nuclear export of viral late mRNAs.

Discussion

The influenza A virus NS1 protein is a polyfunctional molecule that exerts many positive effects on the virus lifecycle though a multitude of interactions with other viral and cellular molecules (32). Here, we characterise a novel functional role of NS1; in promoting the nuclear export of late gene viral mRNAs, in particular M1 but also HA mRNA. We base this conclusion on the notable difference in the bulk localisation of segment 7 mRNA between infected and RNP-transfected cells, a difference that could be obviated by the additional expression of NS1, supported further by the similar alterations in segment 7 mRNA localisation seen in cells infected with NS1-mutant viruses. We also found that NS1 increased the amount of segment 7 mRNA bound by NXF1 and that there was a good correlation between the ability of NS1 mutants to perform this function and to bind NXF1 itself, suggesting that NS1 acts as an adaptor protein that bridges between the viral transcription machinery and the cellular mRNA export pathway. In this model, NS1 replaces (or perhaps augments) the role of the cellular protein Aly

in recruiting the NXF1/p15 complex, thus side-stepping potential blocks to the recruitment of the mRNA export machinery arising from the lack of exon-junction complex deposition or competition between the cellular cap-binding complex and the viral polymerase, both of which are normal routes to attract Aly (17, 18, 43).

Our results are broadly consistent with and complement those recently reported by Mor and colleagues regarding the role of NS1 in the nuclear export of segment 7 mRNAs, with our study focussing on the role of NS1 and theirs focussing more on NS1-mediated intranuclear trafficking of segment 7 mRNA to nuclear speckles for splicing (23). We agree on the point that ultimately, NS1 promotes nuclear export of unspliced M1 mRNA, and extend the finding by showing that both RNA-binding and effector domains of the protein are needed for the activity, which correlates with the ability to act as a bridge between NXF1 and the viral transcript. However, we do not find that this role of NS1 necessarily correlates with a major effect on transcript splicing. While cells infected with the N81 NS1 mutant produced lower levels of mRNA2 than WT virus infected cells (Fig 6), consistent with the effects observed by Mor et al., we did not see a consistent effect of NS1 in a minireplicon setting (Fig 3) and we also show that NS1 promotes nuclear export of the intronless HA transcript (Fig 10).

A role for NS1 in viral mRNA nuclear export is consistent with its known interaction with viral RNPs (44, 45), which would place it in the correct location to interact with a newly synthesised viral mRNA. The association between the viral polymerase and cellular RNA Pol II may also serve to place the nascent viral transcripts in the vicinity for the cellular nuclear export apparatus (14, 46). The interaction of NS1 with the NXF1 export apparatus has also been proposed as a mechanism by which the virus inhibits the export of cellular mRNAs (36). Unlike the mechanism by which NS1 inhibits cellular mRNA export by blocking 3'-end processing and

polyadenylation (47), it is less obvious how targeting the mRNA export apparatus could distinguish viral from cellular mRNAs. One possibility might be via time-dependent effects – an early positive effect on viral mRNA export followed by a late inhibition of the export pathway.

Although NS1 promotes the cytoplasmic accumulation of M1 mRNA, it is not absolutely required, since reduced amounts of cytoplasmic transcripts were still detectable (*e.g.* Fig 2C) and translated even in the absence of the protein (Fig 2D). This is consistent with the fundamental observation that NS1 is a non-essential viral gene in cell culture whose loss nevertheless attenuates virus replication even in interferon-deficient systems (48). The 3-4 fold reduction in M1 accumulation we saw is consistent with the drops in late gene expression noted in many previous studies of NS1 mutants (29, 49-53). The low efficiency export of M1 mRNA in the absence of NS1 may be attributable to direct recruitment of the export apparatus through interactions with the nuclear cap-binding complex (14). A degree of redundancy in the cellular factors recruited for viral mRNA export has precedents from other viral systems (54).

Cell-to-cell variability in nuclear export of segment 7 mRNA in the absence of an intact NS1 protein was also notable. A minority of cells showed cytoplasmic mRNA even in the absence of NS1 (Fig 2B) and NS1 mutants with an overall defect in promoting cytoplasmic accumulation of M1 mRNA produced an altered ratio of cells with apparently normal export to those displaying nuclear retention (Figs 4 and 5), rather than all cells showing an equal reduced efficiency of mRNA cytoplasmic accumulation. The reasons for this are not obvious, but we conjecture that they reflect cell-to-cell variability rather than viral heterogeneity, because we saw similar outcomes from plasmid- and viral-based systems (*e.g.* compare the R+K mutant in Figs 4B and 5B). In some respects, this heterogeneity is reminiscent of the cell-to-cell variability seen in the triggering of innate responses in negative sense RNA virus-infected cells (55). Given the central

role of NS1 in countreracting innate responses and evidence linking this to direct effects on the cellular nuclear export apparatus (32, 36, 56), this is an aspect that warrants further investigation.

Material and Methods

Cells, plasmids, antisera and viruses. Human embryonic kidney 293T cells and human alveolar basal epithelial cells (A549) were cultured as described previously (57). Plasmids pcDNA-PB2, pcDNA-PB1, pcDNA-PA and pcDNA-NP used for the minireplicon assay have been described previously (58) and plasmids pPolI-segment 4, pPolI-segment 7 and pPolI-segment 8 were a gift from Professor Ron Fouchier (59). The NS1 expression vector was kindly provided by Professor Wendy Barclay. NS1 and NS2 genes were PCR cloned into pEGFPN1 in fusion with EGFP using *AgeI* and *KpnI* as restriction sites. NS1 mutants N81, R38A, K41A, R38A+K41A (R+K) and S103/106I (S+I) were produced by site directed mutagenesis (Stratagene). The segment 7 intron was cloned into pcDNA3 (Invitrogen) using *EcoRI* and *HinDIII* sites after PCR amplification of the intron sequence using *Pfu* polymerase (Stratagene). Primer sequences are available on request. GFP-NXF1 plasmid was a gift from Professor Adrian Whitehouse (60). The Cambridge lineage of A/PR/8/34 virus was propagated in embryonated eggs as previously described (61). WT and mutant A/PR/8/34 viruses were rescued in 293T cells using an eight plasmid system (59) as previously described.

Transfection and infection. RNP reconstitution asssays were carried out by transfecting 293T cells with 135 ng of each of plasmids pcDNA-PB2, pcDNA-PB1, pcDNA-PA and pcDNA-NP (3PNP), pPolI Seg 7 and various others as required by the experimental design. At 24 hours post-

transfection (h.p.t.) samples were processed as described previously (58). Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Alternatively 293T cells were infected with wild type A/PR/8/34 and NS1 mutant viruses at an M.O.I of 5 and at 6 h post-infection (h.p.i.) samples were processed as described in (8).

FISH, GFP-Trap pull downs, primer extensions and protein analysis. FISH analysis was performed as described previously (8, 12) at either 24 h.p.t or 6 h.p.i.. RNA probes used to detect segment 7 mRNA were labeled using cyanine 3-UTP (Perkin Elmer) or ChromaTide Alexa Fluor 488-5-UTP (Invitrogen) as previously described (8, 12). GFP-Trap pull down assays were carried out by transfecting 293T cells with 2 µg of a GFP-tagged plasmid (NXF1, WT NS1 or NS1 mutants) and with 250 ng of pEGFPN1 plasmid used as a negative control. Cells were superinfected at 48 h.p.t with either WT A/PR/8/34 or an NS1 mutant virus at an M.O.I of 10 and at 6 h.p.i collected for the pull down assays performed using GFP-Trap beads (Chromatek) as previously described (62). In minireplicon transfections followed by GFP-Trap pull down assays, 500 ng of each plasmid were transfected along with a WT or mutant NS1 GFP-tagged plasmid. The bound fractions of samples were either boiled in SDS-PAGE sample buffer to study protein-protein interactions or used for RNA extraction to analyse protein-RNA interactions. Bound RNA species were identified by reverse transcriptase-radiolabeled primer extension followed by urea-PAGE and autoradiography as described previously (42). Protein analysis was performed by SDS-PAGE and western blotting following standard procedures. Blots were imaged using a Licor Biosciences Odyssey near-infrared imaging platform. Protein quantifications were performed using Licor Odyssey version 3 software.

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450 pGFP-NXF1 and pFlag-NS1 respectively.

451

452 **Figure legends**

453

454 **Figure 1. Localisation of segment 7 mRNA in infected and transfected cells.** 293T cells were
455 (A) infected or mock infected with Cambridge PR8 at an MOI of 5 and fixed at 6 h p.i. or (B)
456 transfected with plasmids to reconstitute RNPs (3PNP) containing segment 7 vRNA or with a
457 negative control set lacking PB2 (2PNP) and fixed 24 h later before staining for positive sense
458 segment 7 RNA by FISH (red) or DNA (DAPI, blue) and imaging by confocal microscopy.
459 Single optical slices are shown. Scale bars: 10 μm.

460

461 **Figure 2. NS1 promotes cytoplasmic accumulation of M1 mRNA.** 293T cells were
462 transfected with plasmids to reconstitute RNPs (3PNP) containing segment 7 vRNA or with a
463 negative control set lacking PB2 (2PNP or -) as well as with other segments or plasmids
464 expressing NS1 or NS2 only or NS1 from influenza B virus (NS1B) and fixed 24 h later before
465 (A, C) staining for positive sense segment 7 RNA (red) or (in C) the intronic sequence of M1
466 (grey) by FISH or DNA (DAPI, blue) and imaging by confocal microscopy. Single optical slices
467 are shown. Scale bar: **10** μm. (B) Individual cells were scored as to whether segment 7 mRNA
468 staining was predominantly nuclear, cytoplasmic or mixed. The mean ± SEM values from 2-5
469 independent experiments are plotted. (D) Cell lysates were analysed by western blotting for the
470 indicated antigens.

471

472 **Figure 3. Accumulation of segment 7 mRNA species in RNP reconstitution assays.** 293T
473 cells were transfected with plasmids to recreate segment 7 RNPs or as a negative control, with a

2PNP combination lacking PB2 either alone (-) or along with other viral components as labelled. 24 h later, total cellular RNA was extracted and (A) analysed by radioactive reverse transcriptase primer extension followed by urea-PAGE and autoradiography with primers specific for segment 7 mRNAs or cellular 5S rRNA. (B) Replicate experiments were quantified by densitometry and data plotted as the mean ± S.D. (n=3) % of total segment 7 mRNA for each of the three species.

Figure 4. Ability of GFP-tagged NS polypeptides to support segment 7 mRNA export. 293T cells were transfected with plasmids to reconstitute RNPs containing segment 7 vRNA or with a negative control set lacking PB2 (2PNP) as well as with plasmids expressing the indicated GFP-tagged proteins and (A) fixed 24 h later before staining for GFP (green), positive sense segment 7 RNA (red) and DNA (DAPI, blue) and imaging by confocal microscopy. Single optical slices are shown. Scale bar: **10** µm. (B) Individual cells were scored as to whether segment 7 mRNA staining was predominantly nuclear, cytoplasmic or mixed. The mean ± SD values from 2-5 independent experiments are plotted. (C) Parallel samples were processed by western blotting for the indicated polypeptides.

Figure 5. Effect of NS1 mutations on segment 7 mRNA localisation in infected cells. 293T cells were infected with the indicated viruses at an MOI of **5** and at 6 h p.i. (A) stained for segment 7 mRNA by FISH (green) and DNA (DAPI, blue) before confocal imaging. Single optical slices are shown. Scale bar: 10 µm. (B) Individual cells were scored as to whether segment 7 mRNA staining was predominantly nuclear, cytoplasmic or mixed. The mean ± SEM values from 3-6 independent experiments are plotted. (C) Cell lysates were analysed by western

blotting for the indicated antigens. (D) M1 and M2 accumulation from replicate experiments was quantified and expressed as a ratio relative to NP expression. Values plotted are normalised to the ratio seen with WT virus and are the mean ± SEM of 3 independent experiments.

Figure 6. Effect of spliceostatin A on segment 7 mRNA localisation. 293T cells were infected with **reverse genetics** PR8 virus at an MOI of **5** and at **6** h p.i. treated or mock treated with **100 µg/ml** spliceostatin A. At 6 h p.i. samples were (A) fixed and stained by FISH for segment 7 mRNA (red) or DAPI for DNA (blue) and imaged by confocal microscopy. Single optical slices are shown. Scale bar = **10** µm. (B) Cell lysates were examined by western blotting for the indicated polyeptides. (C) Total cellular RNA was analysed by radioactive primer extension, urea-PAGE and autoradiography for the indicated RNA species.

Figure 7. Interaction between NXF1 and NS1. 293T cells were transfected with plasmids encoding GFP or GFP-NXF1 and 48 h later either mock infected or infected with the indicated NS1 mutant viruses. Cells were harvested at 6 h.p.i. and cell lysates examined by western blotting for the indicated proteins before (Total) or after (Bound) fractionation over GFP-trap agarose.

Figure 8. Interaction between segment 7 mRNAs and NS1. 293T cells were transfected with plasmids to recreate segment 7 RNPs (3PNP: +) or as a negative control, with a 2PNP combination (3PNP: -) along with the indicated GFP-NS1 polypeptides or with GFP only (GFP-

NS1: -). 48 h later, total cellular RNA was extracted and analysed by radioactive reverse transcriptase primer extension followed by urea-PAGE and autoradiography with primers specific for segment 7 mRNAs or cellular 5S rRNA.

Figure 9. NS1 promotes the interaction of segment 7 mRNA with NXF1. 293T cells were (A) transfected with plasmids to recreate segment 7 RNPs (3PNP: +) or as a negative control, with a 2PNP combination (3PNP: -) along with GFP-NXF1 and with or without NS1 as labelled. 48 h later, total cellular RNA was extracted and analysed by radioactive reverse transcriptase primer extension followed by urea-PAGE and autoradiography with primers specific for segment 7 mRNAs or cellular 5S rRNA. (B) Cells were transfected with GFP-NXF1 (GFP-NXF1: +) or with GFP alone (GFP-NXF1: -) and 48 h later infected or mock infected with the indicated viruses at an MOI of **10.** Total RNA was extracted at 6 h p.i. and analysed as in (A) except that a primer specific for segment 7 vRNA was also included.

Figure 10. Role of NS1 in promoting export of other viral mRNAs. 293T cells were infected or mock infected with the indicated reverse genetics PR8 viruses at an MOI of **5**, fixed at 6 h p.i. and stained for positive sense RNAs from the indicated segments (green) and for DNA (DAPI, blue) before confocal imaging. Single optical slices are shown. Scale bar: 10 µm.

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